Distribution and evolution of ferripyoverdine receptors in *Pseudomonas aeruginosa*

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Summary

Pseudomonas aeruginosa* is a ubiquitous Gram-negative bacterium, which is also able to cause severe opportunistic infections in humans. The colonization of the host is importantly affected by the production of the high-affinity iron (III) scavenging peptidic siderophore pyoverdine. The species *P. aeruginosa* can be divided into three subgroups ('siderovars'), each characterized by the production of a specific pyoverdine and receptor (FpvA). We used a multiplex PCR to determine the FpvA siderovar on 345 *P. aeruginosa* strains from environmental or clinical origin. We found about the same proportion of each type in clinical strains, while FpvA type I was slightly over-represented (49%) in environmental strains. Our multiplex PCR also detected the presence or absence of an additional receptor for type I pyoverdine (FpvB). The *fpvB* gene was in fact present in the vast majority of *P. aeruginosa* strains (93%), regardless of their siderovar or their origin. Finally, molecular analyses of *fpvA* and *fpvB* genes highlighted a complex evolutionary history, probably linked to the central role of iron acquisition in the ecology and virulence of *P. aeruginosa*.

Introduction

Like other ubiquitous aerobic microorganisms, the different *Pseudomonas* species produce siderophores in order to satisfy their need for iron (Braun and Killmann, 1999). *Pseudomonas aeruginosa*, the type species of the genus, is able to thrive in very diverse environments, including water, soil, roots, plant and animal hosts where it is known as an opportunistic pathogen able to cause life-threatening infections (Goldberg, 2000). The common characteristic trait of fluorescent pseudomonads is their capacity to produce, under conditions of iron limitation, the yellow-green fluorescent pigment and siderophore pyoverdine (Meyer, 2000; Ravel and Cornelis, 2003; Cornelis *et al.*, 2007; 2009; Visca *et al.*, 2007). Pyoverdines are composed of a conserved dihydroxyquinoline chromophore, a variable peptide chain, comprising 6–12 amino acids, specific to a producing strain, and a side-chain, generally a dicarboxylic acid or an amide (Ravel and Cornelis, 2003; Visca *et al.*, 2007). Both chromophore (Mossialos *et al.*, 2002) and peptide chain of pyoverdines (Ravel and Cornelis, 2003) are synthesized by non-ribosomal peptide synthetases (NRPSs). A specific TonB-dependent outer membrane receptor recognizes and binds the cognate pyoverdine (Smith *et al.*, 2005). The genes coding for the receptor and the NRPSs responsible for the synthesis of the peptide moiety of pyoverdine are part of the so-called 'variable' locus of pyoverdine genes (Ravel and Cornelis, 2003; Smith *et al.*, 2005; Cornelis *et al.*, 2007; Visca *et al.*, 2007). Three siderovars of *P. aeruginosa* can be distinguished, producing three structurally different types of pyoverdine (type I, II, III) (Cornelis *et al.*, 1989; Meyer *et al.*, 1997; De Vos *et al.*, 2001; Ernst *et al.*, 2003; Spencer *et al.*, 2003; Smith *et al.*, 2005), each being recognized at the level of the outer membrane by a specific receptor (Cornelis *et al.*, 1989; De Chial *et al.*, 2003; Spencer *et al.*, 2003). It has also been shown that the type II ferripyoverdine receptors are more diverse and it has been suggested that the type II receptor gene is under positive selection (Smith *et al.*, 2005; Tümmler and Cornelis, 2005). This selection pressure could be due to the pyocin S3 bacteriocin which uses type II ferripyoverdine receptors in order to enter the cell and kill it (Baysse *et al.*, 1999; De Chial *et al.*, 2003). However, another pyocin, S2, was recently found to kill strains having the type I FpvA receptor, which does not

show such variability, contradicting this hypothesis (Denayer *et al.*, 2007). A second receptor specific for type I pyoverdine, called FpvB, the gene of which is not part of the pyoverdine locus, has also been identified (Ghysels *et al.*, 2004). The *fpvB* gene was also detected in other *P. aeruginosa* strains, including some that produce type II and type III pyoverdines, where it was found to confer the capacity to utilize type I pyoverdine as a source of iron (Ghysels *et al.*, 2004). Here, using a multiplex PCR (MPCR) approach, we found a slightly different proportion of each pyoverdine receptor type between clinical and environmental strains and report that the *fpvB* gene is almost ubiquitous among *P. aeruginosa* strains. Moreover, sequencing and molecular analyses of *fpvA* and *fpvB* genes from each *P. aeruginosa* siderotype highlighted a complex evolutionary history.

Results

Existence of fpvA *type II variants*

With the previously developed MPCR method for identification of *fpvA*I, II and III receptor genes in *P. aeruginosa* (De Chial *et al.*, 2003) we failed to amplify an *fpvA* fragment in some isolates known to produce type II PVD (as evidenced by IEF typing of pyoverdines), including the type II reference strain ATCC 27853. Spencer and colleagues (2003) described a new FpvA receptor sequence (Accession No. AAO1728) and *in silico* analysis indicated that this receptor is a variant of the FpvAII receptor that we previously described (Accession No. AAN62913) (De Chial *et al.*, 2003). At the nucleotide level, both genes share 89% of the residues in an overlap of more than 90% of their sequence. We therefore designed a primer set for the specific amplification of a fragment of this *fpvA*II gene variant and detected its presence (PCR detection) in *P. aeruginosa* ATCC 27853 and other type II *P. aeruginosa* strains that failed to give amplification with the previously designed MPCR primer set (De Chial *et al.*, 2003). We therefore called this second type II receptor '*fpvA*IIb' and the original type II receptor from 7NSK2 '*fpvA*IIa'.

Multiplex PCR for the simultaneous detection of five P. aeruginosa *ferripyoverdine receptor genes*

Previously, we reported the presence of a second type I ferripyoverdine transport mediating receptor in *P. aeruginosa* PAO1, encoded by *fpvB* (PA4168) (Ghysels *et al.*, 2004). We also demonstrated the presence of functional *fpvB* homologues in type II and type III *P. aeruginosa* strains (Ghysels *et al.*, 2004). A primer set for *fpvB* detection and one for detection of *fpvA*IIb were therefore added to the original MPCR primer set for detection of *fpvA*, *fpvA*IIa and *fpvA*III (De Chial *et al.*, 2003). With this five-
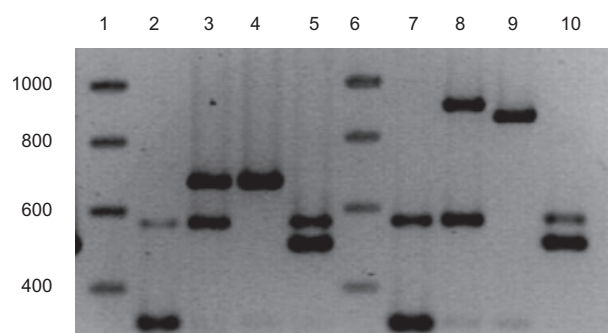

Fig. 1. Multiplex PCR-amplified fragments of four reference strains (PAO1: lanes 2 and 7, 7NSK2: lanes 3 and 8, ATCC 27853: lanes 4 and 9, and 59.20: lanes 5 and 10) with two different primer sets, electrophoretically separated. The first and sixth lanes contain the molecular weight markers (sizes in bp indicated on the left). The bands corresponding to the different PVD receptors have the following sizes in reverse order of size for set 1 (lanes 2–5): *fpvA*II, for both variants (682 bp), *fpvB* (562 bp), *fpvA*III (505 bp) and *fpvA*I (324 bp), for set 2 (lanes 7–10): *fpvA*IIa (908 bp), *fpvA*IIb (863 bp), *fpvB* (562 bp), *fpvA*III (505 bp) and *fpvA*I (324 bp).

primer-pair MPCR set, we were able to detect simultaneously five different ferripyoverdine receptor genes in different *P. aeruginosa* strains, two in the PVD type reference strain PAO1 (*fpvA*I and *fpvB*), two in the type IIa reference strain 7NSK2 (*fpvA*IIa and *fpvB*), one in the type IIb reference strain ATCC 27853 (*fpvA*IIb) and two in the type III reference strain 59.20 (*fpvA*III and *fpvB*) (Fig. 1). We also used an MPCR primer set in which the primers for detecting *fpvA*IIa and *fpvA*IIb are replaced by a single primer pair which detects both genes without discrimination (Fig. 1). It is important to note that even with the bacterial cells directly inoculated as template in the PCR-mix (without prior boiling), clear amplifications were obtained.

Distribution of ferripyoverdine receptor genes in a P. aeruginosa *population*

The MPCR described above was applied to study the distribution of the currently identified ferripyoverdine receptor genes in a *P. aeruginosa* population comprising 345 clinical and environmental isolates from different locations throughout the world. The results are summarized in Table 1 and the complete list of strains with their origin is given in Table S1 in *Supporting information*. From only four isolates (1.2%) no amplification signal could be detected, while all the other strains were positive for at least one receptor gene (Table 1).

From these 341 MPCR-positive isolates, 122 (35.8%) had *fpvA*I, 48 had *fpvA*IIa (14.1%), 80 (23.5%) had *fpvA*IIb and 83 (24.3%) had the *fpvA*III gene, while in eight strains (2.3%) only *fpvB* could be amplified. It is important to note that the distribution is slightly different according to the

Table 1. Results of the multiplex PCR of 345 *P. aeruginosa* strains.

Positive strains	*fpvA*I	*fpvA*IIa	*fpvA*IIb	*fpvA*III	*fpvB*
5	+				
117	+				+
2		+			
46		+			+
5			+		
75			+		+
8				+	
75				+	+
8					+
4					

origin of the strains (Table S1). The clinical strains (220 strains) showed about the same proportion of each type, while FpvA type I was over-represented (49%) in environmental strains (79 strains).

Altogether *fpvB* was amplified from 317 strains (93%) either alone or together with *fpvA*I, *fpvA*IIa, *fpvA*IIb or *fpvA*III. The *fpvB* gene could not be amplified in 4.1%, 4.2%, 6.3% and 9.6% of the strains that were positive for *fpvA*I, *fpvA*IIa, *fpvA*IIb or *fpvA*III respectively. Figure 2 shows a similarity tree based on AFLP patterns, sequences of *oprI*, *oprL* and *oprD*, and serotypes. Results of the MPCR are also shown for each strain. All 75 strains in the tree are mentioned in Table 1 and Table S1, except LMG 10643, which is not a *P. aeruginosa*, but a *Pseudomonas oryzihabitans*.

Comparison between IEF pyoverdine determination and receptor typing

Isoelectrofocalization of pyoverdines from the spent medium is a technique allowing fast and accurate determination of the pyoverdine type in *P. aeruginosa* (Meyer *et al.*, 1997; De Vos *et al.*, 2001). However, some strains had lost the ability to produce pyoverdine, as evidenced in some cystic fibrosis isolates, but were still able to take up ferripyoverdine (De Vos *et al.*, 2001; Ernst *et al.*, 2003). For these pyoverdine-negative mutants, growth stimulation experiments with purified pyoverdines did not provide clear-cut answers because of the ability of some strains to utilize more than one type of ferripyoverdine as a source of iron (De Vos *et al.*, 2001; Ghysels *et al.*, 2004). This is due to the presence of FpvB, the alternative receptor for type I ferripyoverdine and also because the type III ferripyoverdine receptor also allows some level of utilization of the type II ferripyoverdine (Ghysels *et al.*, 2004). All pyoverdine-positive strains, which were tested by IEF, showed the same *fpvA* receptor type as the corresponding pyoverdine, in addition to the presence or absence of *fpvB* gene (results not shown). Four of the *fpvA*-negative strains (So122, Lo059, Pr332 and Br700 strains) were found to produce type II pyoverdine, suggesting the exist-

ence of further type II receptor variants while the others were pyoverdine-negative (Table S1).

Functionality of the fpvB gene

In some pyoverdine-negative strains *fpvB* was amplified, either singly or together with an *fpvA* gene. The results presented in Fig. 3 for strains Mi159 and Mi162 show that *fpvB* is expressed and functional as judged by the growth stimulation assay using the three purified pyoverdines. Both isolates are pyoverdine-negative, but in Mi159 both *fpvA*III and *fpvB* were amplified by PCR and only *fpvB* in Mi162. The growth of Mi159 was stimulated by the three pyoverdines, showing a good correlation with the presence of FpvAIII and of FpvB. As already mentioned, FpvAIII allows the uptake not only of type III, but also, to some extent, of type II ferripyoverdine, and FpvB is responsible for the uptake of type I ferripyoverdine (Ghysels *et al.*, 2004). In Mi162 only FpvB seems to be functional.

In strains SG17M, C2 and C19, which can be typed by IEF as type II pyoverdine producers (although their pyoverdine production is low), both *fpvA*IIb and *fpvB* can be amplified, although only in the case of SG17M could the growth be stimulated by type I pyoverdine, indicating either that in C2 and C19 the *fpvB* gene is not expressed or that its product is not functional (results not shown).

Nature of a supplementary 450 bp PCR fragment detected in some P. aeruginosa strains

In a minority of the strains (8.9%) we obtained, in addition to the expected fragment associated with the different *fpvA* receptors, an additional amplicon of around 450 bp (Fig. 4). Closer analysis revealed that this fragment was the PCR product of the primer pair *fpvA*If and *fpvB*f. A BLASTX search of *Pseudomonas* genomes revealed that the translated product had 94% identity with the products of two genes from PA7, PSPA7_0713 and PSPA7_5043 which are annotated as coding putative phage proteins. The fragment also appeared to be more frequently amplified in type III strains (18%) than in type II (9%) and type I strains (2.5%).

Phylogeny of PVD receptors

In order to investigate the evolutionary history of the ferripyoverdine receptor genes in *P. aeruginosa*, we carried out a phylogenetic analysis with 8 *fpvA*I, 10 *fpvA*II (4 IIa and 6 IIb), 8 *fpvA*III and 15 *fpvB* genes from 22 strains (Fig. 5A). While the dendrogram shows a great variability between *fpvA* and *fpvB* clusters, the variability within each *fpvA* and *fpvB* cluster is much lower, as highlighted by the scales on the dendrograms and the overall mean variabil-
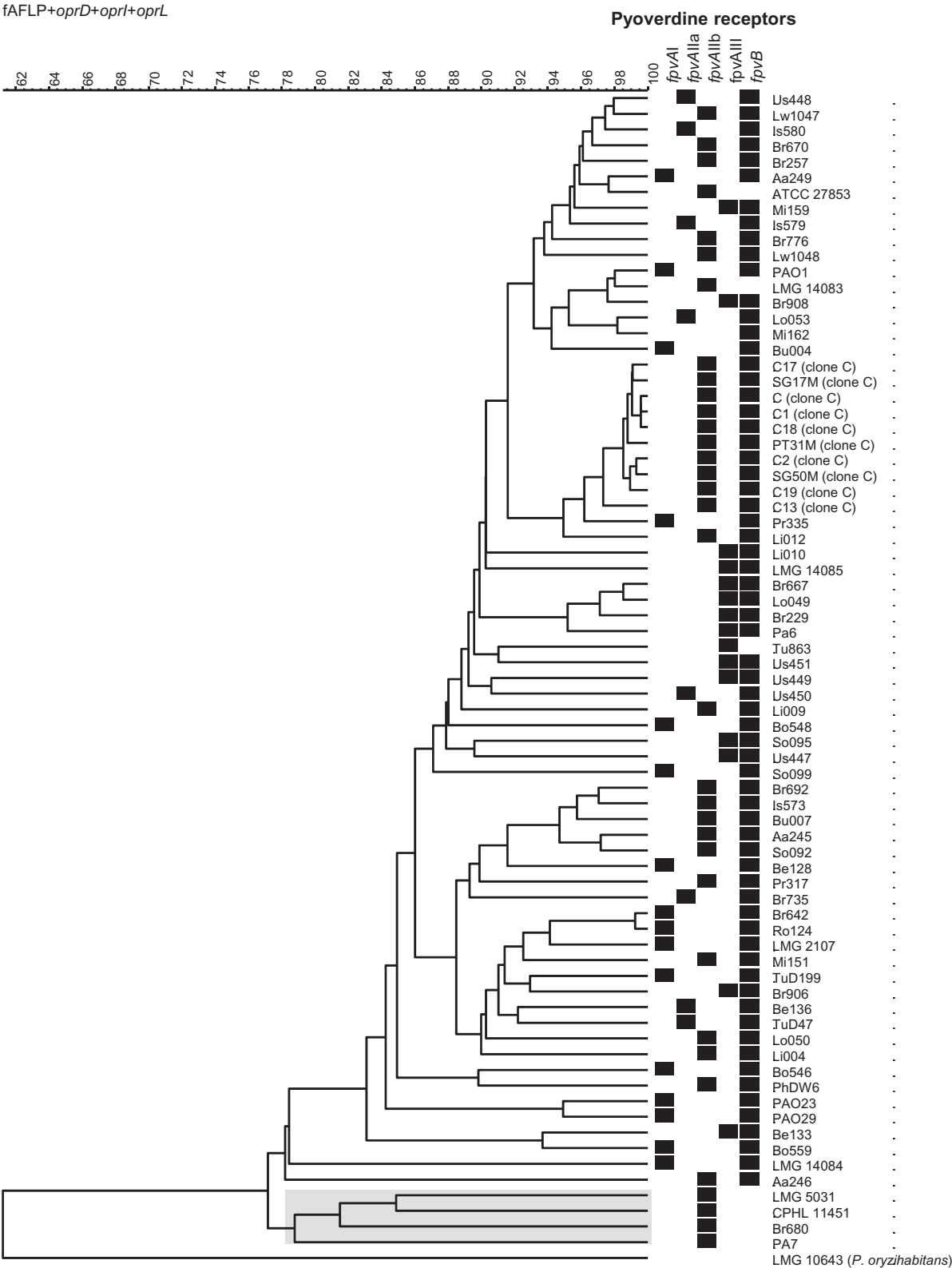
fAFLP+*oprD*+*oprI*+*oprL*

Pyoverdine receptors

Fig. 2. Dendrogram (UPGMA, BioNumerics v5.2) based on the comparison of the composite data set consisting of the AFLP pattern, the *oprI*, *oprL* and *oprD* nucleotide sequences and the serotype of 75 diverse *P. aeruginosa* strains isolated from different clinical and environmental sites across the world. Black squares represent the type of receptor identified by MPCR. Strain name, geographical origin, isolation site and year and pyoverdine receptor profiles are shown in Table S1. The PA7 clade is highlighted in grey.
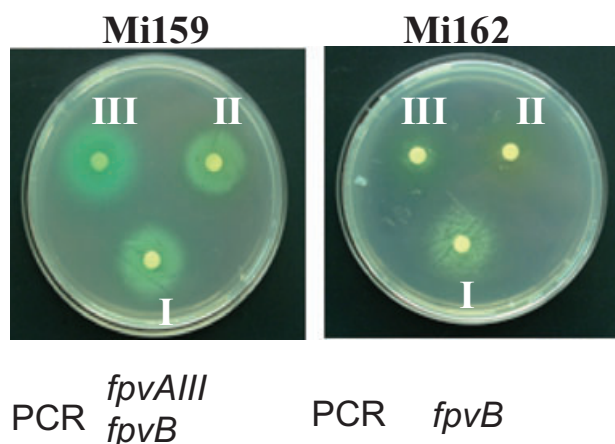
Mi159 Mi162

PCR   *fpvAIII*
        fpvB

PCR   *fpvB*

Fig. 3. Result of growth stimulation assays using disks impregnated with 2 mM purified pyoverdines (types I–III as indicated on the picture) for strains Mi159 and Mi162. The insert below shows the result of the multiplex PCR amplification.

ity (Pi) in Table 2. Among these clusters, the *fpvA*II cluster is the most discriminatory (Pi is about twofold higher), with two robust subclusters (Bayesian posterior probabilities ≥ 98%). The other *fpv* clusters contain more similar sequences revealing ambiguous topologies with statistical supports frequently lower than 50%.

To explain the presence of these three very different types at the *fpvA* locus, it seems likely that some lateral transfers have occurred during the evolutionary history of the ferripyoverdine receptor genes of *P. aeruginosa*. Accordingly, several articles already suggested that several lateral transfers occurred in *P. aeruginosa*, especially at pyoverdine locus (Pirnay *et al.*, 2005; Smith *et al.*, 2005; Wiehlmann *et al.*, 2007).

In order to get insight into the evolutionary history of pyoverdine genes, we used several approaches. First, we carried out a comparative analysis between the ferripyoverdine receptor gene and organism phylogenies. Second, we looked at the synonymous codon usage [codon adaptation index (CAI index)] and the GC content.

Comparison between PVD receptor and organism phylogenies

We investigated the evolutionary history of organisms in a fine resolution by using a dendrogram (UPGMA, BioNumerics v5.2) based on the comparison of the composite data set consisting of AFLP patterns, *oprI*, *oprL* and *oprD* nucleotide sequences and serotypes (Fig. 2). In general, we can see that the closely related strains (i.e. with a similarity superior to 85%) presented the same *fpvA*/*fpvB* distribution, especially when the strains were epidemiologically related (e.g. clone C) but also when they were not (e.g. Br692 versus Is573 strains). In contrast, some

closely related strains (e.g. PAO1 versus LMG14083 strains or Lo053 versus Mi162 strains) showed different *fpvA*/*fpvB* distribution, corresponding likely to some lateral transfers. Because the profiles become probably too different when the strains are not closely related, almost all the dendrograms constructed from molecular fingerprints lose resolution in the deeper nodes.

In order to compensate for this putative limitation, we estimated an organism phylogeny at a larger resolution by using a set of 34 ribosomal concatenated genes from seven sequenced *P. aeruginosa* genomes (Fig. 5B). Interestingly, while the topology between six closely related strains (less than 0.3% of difference) has not been fully resolved (some weak statistical supports), the PA7 strain is well separated from the other strains in the organism phylogeny (maximum Bayesian posterior probabilities). We cannot formally exclude a faster evolution of the PA7 strain, as happens with a mutator strain. However, the position of the root as highlighted by out-grouping with *Pseudomonas mendocina* and *Azotobacter vinelandii* shows clearly an early divergence of this strain in the *P. aeruginosa* species (Fig. 5B). Moreover, from the last *P. aeruginosa* common ancestor, about the same evolutionary distance is observed to each strain.

Because both phylogenies (of ferripyoverdine receptor genes and concatenated ribosomal genes) have not been fully resolved, it is difficult to compare them. However, two observations can be made. (i) The presence of the same
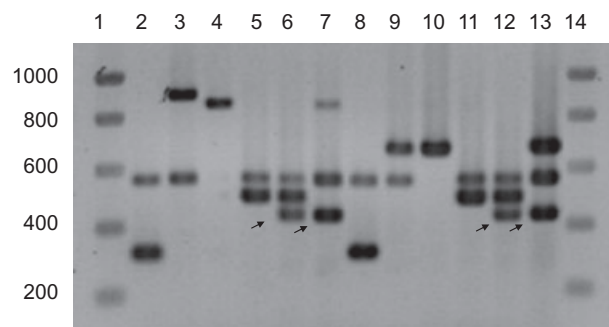

Fig. 4. Multiplex PCR-amplified fragments of six strains with two different primer sets, and separated by electrophoresis on 1% agarose. The first and 14th lanes contain the molecular weight markers as in Fig. 1. The bands corresponding to the different PVD receptors have the following sizes in reverse order of molecular weight for set 1 (lanes 2–7): *fpvA*IIa (908 bp), *fpvA*IIb (863 bp), *fpvB* (562 bp), *fpvA*III (505 bp) and *fpvA*I (324 bp), for set 2 (lanes 8–13): *fpvA*II, for both variants (682 bp), *fpvB* (562 bp), *fpvA*III (505 bp) and *fpvA*I (324 bp). The four pyoverdine type reference strains are: PAO1 (lanes 2 and 8), 7NSK2 (lanes 3 and 9), ATCC 27853 (lanes 4 and 10) and 59.20 (lanes 5 and 11). In strains Br667 (lanes 6 and 12) and Is573 (lanes 7 and 13) we amplified, in addition to the bands corresponding to their receptor types (which is *fpvA*III and *fpvB* for Br667 and *fpvA*IIb and *fpvB* for Is573), another band of around 450 bp (indicated by arrows) which appeared to be the result of amplification of a genomic fragment, probably of phage origin that is present in a small fraction of the *P. aeruginosa* population.
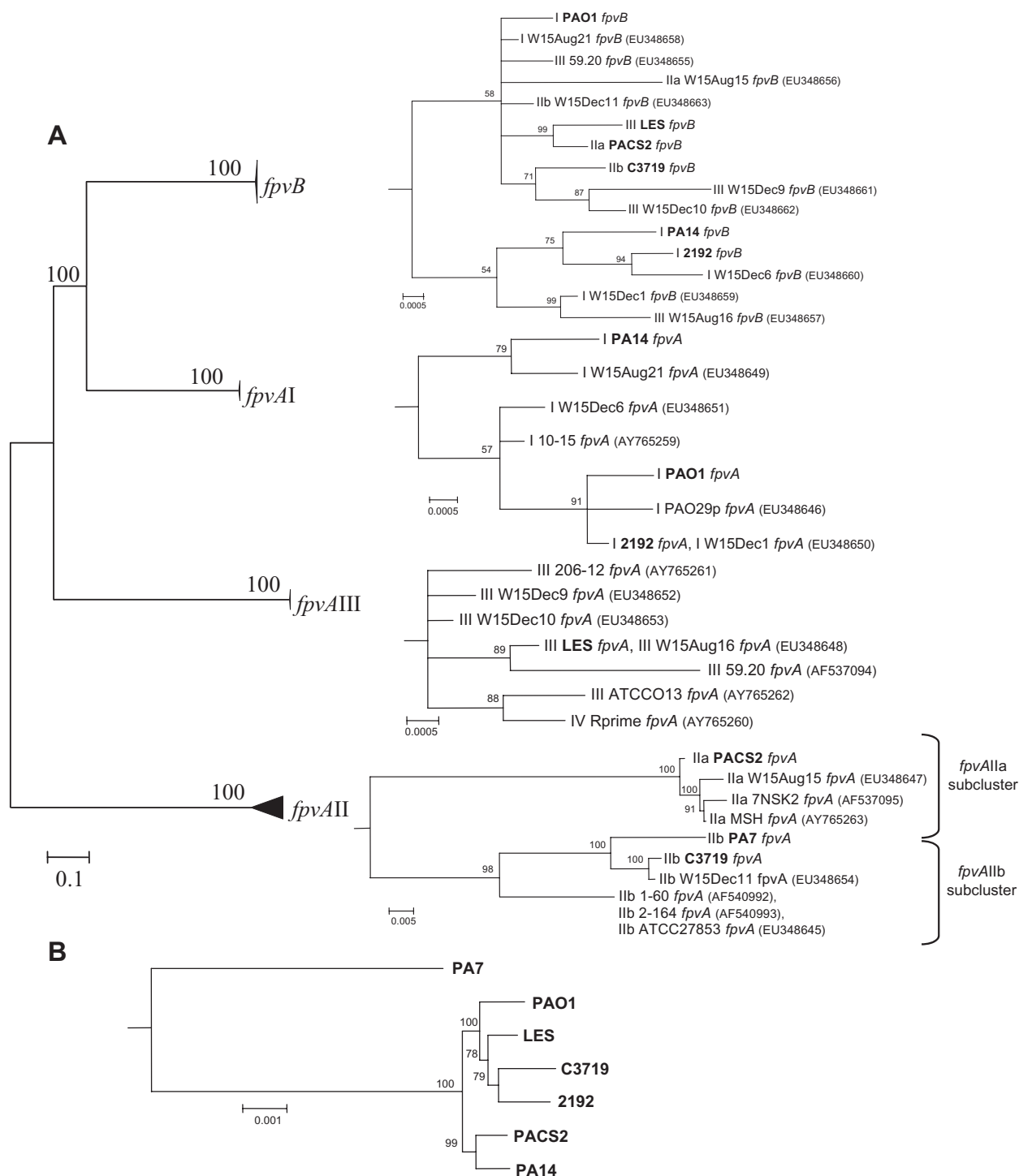
Fig. 5. Phylogenetic relationships among ferripyoverdine receptor genes (A) and 34 concatenated ribosomal genes (B) from *P. aeruginosa* isolates. Strains for which the genome is sequenced are in bold. For the phylogenetic tree of ferripyoverdine receptor genes (A), each cluster (*fpvA*I, *fpvA*II, *fpvA*III, *fpvB*) is highlighted separately, because of a great difference between intra- and inter-cluster variabilities. The names of the sequences on the subtrees correspond, respectively, to the *fpvA* type (I, IIa, IIb or III) of the corresponding strain, the strain name, the gene name (*fpvA* or *fpvB*), and the GenBank accession number (expect for genes from sequenced genome). For the phylogenetic tree of 34 concatenated ribosomal genes (B), the position of the root was determined by using *P. mendocina* and *Azotobacter vinelandii* (ymp and AvOP strains respectively) as out-group. All the dendrograms were generated using Bayesian inference under a GTR +γ model of evolution. Numbers on tree branches report Bayesian posterior probabilities (expressed in percentage). Only statistical support ≥ 50% are reported (majority rule consensus tree). The horizontal length of branches is proportional to the estimated number of substitutions. For each tree, the scale corresponds to the number of substitutions per site.

Table 2. Properties of the genes analysed in this study.

Set of genes	Number of strains[a]	Length of sequences (bp)	GC content (mol%)	CAI	Overall mean variability (Pi)	Mean K_s (K_a/K_s)
Concatenated ribosomal genes[b]	7	12 735	59.7–59.9	0.77	0.006	0.017 (0.060)
*fpvA*I	7	2 367	60.8–61.2	0.81–0.82	0.003	0.009 (0.034)
*fpvA*II	8	2 382	62.2–64.0	0.83–0.87	0.073	0.161 (0.253)
*fpvA*III	7	2 265	59.4–59.6	0.76	0.003	0.007 (0.159)
fpvB	15	2 343	66.1–66.7	0.86–0.87	0.005	0.011 (0.229)

a. See the name of strains in Fig. 5A. Identical sequences were removed for this analysis.
b. Corresponding to 34 concatenated ribosomal genes (see *Experimental procedures*).

fpvA type (IIb) in two evolutionary-distant strains (PA7 and C3719 strains) added to the presence of all the possible *fpvA* types in closely related strains (PAO1, C3719, LES, 2192 and PACS2) confirmed that some lateral transfers have likely occurred at the *fpvA* locus. (ii) The second observation concerns the presence or absence of the *fpvB* gene in the seven sequenced genomes. Since the *fpvB* gene was detected in about 93% of our set of 345 *P. aeruginosa* strains and not in other *Pseudomonas* species (even in the close species *P. mendocina*), it might be useful to know whether the insertion of the *fpvB* gene was correlated with the *P. aeruginosa* speciation event, followed by some deletion events, or whether the insertion of the *fpvB* gene occurred after the speciation event, highlighted by an ancestral state of some *P. aeruginosa* strains without the *fpvB* gene. Interestingly, the peculiar PA7 strain is the only strain with a sequenced genome without the *fpvB* gene. Moreover, *fpvB* was not detected in three other strains forming a cluster with the PA7 strain (denominated 'PA7 clade' as highlighted in Fig. 2) in the composite dendrogram analysis. Since these three strains were not temporally and spatially related, we wondered whether the *fpvB* insertion event occurred after the divergence of the PA7 clade, which could have inherited the ancestral state without the *fpvB* gene. An alternative hypothesis would be a lateral transfer of *fpvB* gene before the divergence of the PA7 clade, followed by a deletion after this divergence. In both scenarios, the *fpvB* gene could have been introduced just before, during or just after the speciation event. It is worth to mention again that all strains in PA7 clade had the *fpvA*IIb gene. By studying the genomic context of the *fpvB* gene (http://v2.pseudomonas.com), it can be observed that the regions upstream and downstream of *fpvB* are conserved in PAO1, LES and PA14. These genomic regions are also conserved in the genome of PA7 strain (Fig. 6). Interestingly, in PA7, the two genes flanking *fpvB* are conserved, and in place of *fpvB* a fragment of about 100 nucleotides showing 93% of identity with the end of the gene can be detected, highlighting an ancient deletion of the *fpvB* gene in the PA7 clade.

Study of the synonymous codon usage and the GC content

Finally, we studied the synonymous codon usage (CAI index) and the GC content to investigate the occurrence of lateral transfers during the evolutionary history of the ferripyoverdine receptor genes in *P. aeruginosa*. As expected, for each gene or set of genes, the CAI index
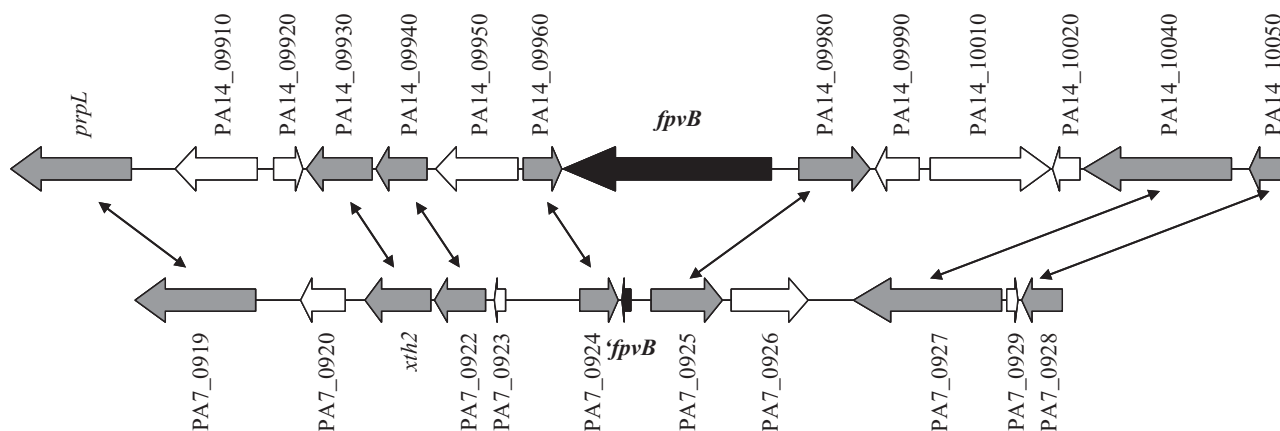

Fig. 6. Schematic representation of the genomic region around the (complete or partial) *fpvB* gene in the PA14 (above) and PA7 (bellow) strains. ORFs oriented in the right are in the leading strand. Orthologous genes (according to the Pseudomonas Genome Project) are in grey and linked by double arrows.

and GC content were conserved between the sequenced *P. aeruginosa* strains for a given gene, while these features varied between the genes for a given strain (Table 2). However, it should be noted that, again, the PA7 strain had the highest value of GC content and CAI index for ribosomal genes and the *fpvA*IIb gene. When comparing the GC content of *fpvA* genes we can see a decrease in % GC from *fpvA*II to *fpvA*III (Table 2). Since many driving forces are responsible for variations in GC content (e.g. position in the genome) or in synonymous codon usage (e.g. level of gene expression), it is usually difficult to compare these features between different genes. However, as expected, the concatenated ribosomal genes showed a GC content of about 60%, a classically lower value than the GC content calculated from the core genome of PAO1 strain (67.1%) (Wolfgang *et al.*, 2003; Bodilis and Barray, 2006). Interestingly, the *fpvB* gene showed the same GC content as for the core genome and high CAI values, typical for a gene present in the lineage for a long time. These features are in agreement with both the evolutionary scenario described above, suggesting that the *fpvB* gene was introduced early in the *P. aeruginosa* lineage and subsequently lost in some strains such as those representing the PA7 clade. Concerning the *fpvA* genes, since the different alleles are roughly at the same locus, code a similar function and present *a priori* the same level of expression, we could expect the same CAI index and GC content values between the three *fpvA* types. Because this was not the case, we deduced that some inter-species lateral transfers occurred at different times and/or from different organisms, lateral transfer of the *fpvA*II gene being the more ancient event and/or from a closely related organism, followed by the *fpvA*I gene and finally the *fpvA*III gene.

The fpvAIII *gene of LES strain is triplicated*

Analysis of the recently annotated genome of the Liverpool epidemic strain (LES) revealed that three identical copies of *fpvA*III are present (http://www.pseudomonas.com). Also the *pvdE* gene is triplicated and there are two incomplete *pvdF* genes before each *fpvA*III. The sequences of *pvdE* and *fpvA*III are all identical to each other.

Discussion

Because most of the studies about the population structure of *P. aeruginosa* had an epidemiological goal and focused on recent clonal expansions, geographic localizations and links with virulence factors and pathogenicity, little was known about the early evolutionary history of the *P. aeruginosa* species. In this article, we have approached this aspect in order to study the distribution of ferripyover-

dine receptor genes from an evolutionary point of view. We therefore estimated an organism phylogeny in the scale of the *P. aeruginosa* species from parts of the core genome of the seven *P. aeruginosa* sequenced genomes. The phylogenetic tree obtained from ribosomal genes showed an early divergence of PA7 strain that was strongly distant from the six other closely related *P. aeruginosa* strains (Figs 2 and 5B). These six strains were not clearly evolutionarily distinct from each other, with a not fully supported topology, probably because of a very limited variability. In contrast, a composite dendrogram (including AFLP pattern, *oprI*, *oprL* and *oprD* gene sequences, and serotype) was useful to discriminate between those more closely related strains but may have some limitations on a larger scale. Altogether, the use of these two phylogenic approaches permitted us to study the evolutionary history in the whole *P. aeruginosa* species.

The ribosomal genes have already been shown to be useful for constructing a robust phylogeny among *Pseudomonas* (Bodilis and Barray, 2006). It is important to note that there are some discussions about methods for estimating phylogeny from a set of genes (Gadagkar *et al.*, 2005). Phylogeny could be estimated either from concatenated genes (as we did), or by carrying out a consensus from individual trees. The principal argument against phylogeny from concatenated genes is the variation of the evolutionary rate between functionally distinct genes. However, because the ribosomal genes code for functionally linked proteins and have likely evolved slowly at the same evolutionary rate (independent of environmental changes), we argue that this argument against phylogeny from concatenated genes is not valuable here. Second, from the 34 (generally not well supported) trees constructed from individual ribosomal genes, we arrived to the same conclusions, i.e. a strong separation of the PA7 strain and variable topologies for the six other closely related strains (data not shown).

Pseudomonas aeruginosa is a ubiquitous microorganism, which is endowed with a high capacity for adaptation to different niches (Goldberg, 2000). This is reflected in its capacity to take up different siderophores next to the uptake of its own siderophores, pyoverdine and pyochelin (Cornelis and Matthijs, 2002; Cornelis *et al.*, 2007; 2009). *Pseudomonas aeruginosa* strains can be subdivided into three groups based on the type of pyoverdine they produce (Cornelis *et al.*, 1989; Meyer *et al.*, 1997; De Vos *et al.*, 2001). The receptors corresponding to these three ferripyoverdines have now been identified by different teams (Poole *et al.*, 1993; De Chial *et al.*, 2003; Spencer *et al.*, 2003; Smith *et al.*, 2005). Here, by using an MPCR, we typed 345 clinical and environmental isolates from different locations throughout the world and found a similar distribution of each

receptor type, type I being slightly over-represented in environmental strains. Interestingly, in a recent work on 240 *P. aeruginosa* strains (only a few strains were common with our study), Wiehlmann and colleagues (2007) found about the same proportion for each ferripyoverdine receptor type. Since competition for iron plays an important role for the fitness of *Pseudomonas* (Griffin *et al.*, 2004) a link between the distribution of the FpvA types and the ecological niches could be expected. The slightly different proportion of each type between environmental and clinical strains would be interesting to investigate further by studying the coexistence of strains with different FpvA types, in terms of cooperation (which would tend to limit the number of different PVD type) and competition (which would tend to increase the number of different PVD type).

Another important observation concerns the conservation of the *fpvB* gene among *P. aeruginosa* strains, suggesting that the ability to utilize type I ferripyoverdine as a source of iron is a common trait of the vast majority of *P. aeruginosa* strains (Ghysels *et al.*, 2004). Although we did not investigate the functionality of FpvB in a large number of strains, it is evident that there are some instances where the gene is present (or at least the part we amplified with the primers used in this study), but the ability to utilize the heterologous type I pyoverdine could not be observed, perhaps because *fpvB* is not expressed in these strains. In the study of Wiehlmann and colleagues (2007), the authors found that 10% of the *P. aeruginosa* tested do not have the *fpvB* gene, which is close to the 7% we found. Moreover, it could be deduced from both the study of Wiehlmann and colleagues (2007) and ours (Fig. 2) that at least a few deletions of *fpvB* genes have occurred as evidenced in PA7 (Fig. 6). Because *fpvB* was only found in *P. aeruginosa* and was absent in other *Pseudomonas* spp., we formulate the hypothesis of an ancestral state of some *P. aeruginosa* strains before the insertion of the *fpvB* gene. So, the *fpvB* gene was likely introduced early in the *P. aeruginosa* species (or just before the speciation event), and lost in the PA7 clade. The deletion of *fpvB* would therefore have occurred in the PA7 clade soon after its insertion. This observation refutes thus the most parsimonious hypothesis of an ancestral state without *fpvB* inherited by the PA7 clade.

Finally, the fact that the great majority (more than 90%) of *P. aeruginosa* have *fpvB* could highlight a fundamental role of this gene in the ecology of this species. Nevertheless, it cannot be excluded that introduction of *fpvB* in the *P. aeruginosa* species would be concomitant with a transfer of a more important gene and so, would result from a genetic hitchhiking.

In their interesting study on the evolution of pyoverdine biosynthesis and uptake genes, Smith and colleagues (2005) propose that the pyoverdine region has been acquired by horizontal transfer, since the codon usage of the corresponding genes is unusual. Within the *P. aeruginosa* pyoverdine region, some genes show high divergence between types. These genes include the NRPS genes involved in the biosynthesis of the pyoverdine peptide chain, the *pvdE* gene coding for an ABC transporter, and the *fpvA* gene encoding the receptor (Ravel and Cornelis, 2003; Smith *et al.*, 2005; Visca *et al.*, 2007). Based on large strain collections, this study and two previous studies (Pirnay *et al.*, 2005; Wiehlmann *et al.*, 2007) have arrived at the same conclusion of frequent intra-species lateral transfers of *fpvA* genes, correlated with the important role of the FpvA type in the fitness of *P. aeruginosa*. It is interesting to mention that in other fluorescent pseudomonads the genes involved in the biosynthesis and uptake of pyoverdine are also clustered, suggesting that horizontal gene transfers have also occurred in these species (Ravel and Cornelis, 2003). According to the study of Smith and colleagues (2005), from GC content and synonymous codon usage it seems that the type III ferripyoverdine receptor gene was transferred more recently or from a more distant organism than the other two types, in agreement with the low GC content of this gene (59%), the lowest of all other TonB-dependent receptor genes, which have an average value of 67% (P. Cornelis and J. Bodilis, in preparation). In contrast, the type IIb ferripyoverdine receptor gene was probably transferred before the other two types or from a more closely related organism. Interestingly, since FpvAIIb is the receptor of the peculiar PA7 clade, it may be the first *fpvA* type of the *P. aeruginosa* species.

Intra-type variability and tests for positive selection have highlighted a diversifying selection of the *fpvA*II gene (Smith *et al.*, 2005; Tümmler and Cornelis, 2005). Smith and colleagues (2005) made the suggestion that the more rapid evolution of this gene might be driven by the need to resist killing by pyocin S3, for which FpvAII is the receptor (Baysse *et al.*, 1999; De Chial *et al.*, 2003). Although we also think that a Darwinian selection most likely occurred for the *fpvA* gene, we do not totally agree with this hypothesis of driving force proposed by Smith and colleagues (2005). First, we have recently shown that another soluble pyocin, S2, kills strains having the type I ferripyoverdine receptor, but sequences of different *fpvA*I alleles from S2-sensitive and S2-resistant strains did not reveal such a diversifying selection (Denayer *et al.*, 2007). The second argument is the sensitivity to pyocin S3 of strains with both FpvAII receptor subtypes (IIa and IIb), highlighting that this positive selection gives no particular advantage for resistance to pyocin S3 (data not shown). So, the driving force may be unknown yet, e.g. the use of FpvAII as a phage receptor or the need to escape to the immune system. To

explain this observed positive selection and more generally to explain the great diversity of the PVD/FpvA pairs, we suggest an alternative scenario where the evolution of the receptor is driven essentially by changes in pyoverdine structure. In the competition for iron, new pyoverdine structures could offer a selective advantage. In this context, we hypothesize that the changes occur first in just one or only a few modules of the NRPS for the biosynthesis of a given pyoverdine. Since a receptor can sometimes recognize heterologous pyoverdines (Ghysels *et al.*, 2004), a new pyoverdine variant could still be recognized by the receptor, although with lower efficiency. This could now drive the evolution of the receptor towards a finer specificity, by a positive selection. In this scenario, the type II pyoverdine would result from relatively recent modifications in its structure (in fact, perhaps concomitant with the speciation event) and the recognition of the pyoverdine by the receptor would not yet be optimized. In this regard, it is important to mention that type II FpvA is the receptor showing the highest specificity, since it does not allow the transport of the other two *P. aeruginosa* pyoverdines (Ghysels *et al.*, 2004). In order to check this hypothesis, it would be interesting to study the competition between bacteria with type IIa and those with type IIb FpvA in conditions of iron limitation, with or without pyocin S3. Since evolution of receptors could also be facilitated by gene duplications, it is of interest to notice that three copies of *pvdE* and *fpvA*III exist in the LES strain. However, the three copies are identical, suggesting that this is a recent event. In *Pseudomonas syringae* genomes there are two copies of *fpvA* in tandem, but the two proteins are only 73% identical (P. Cornelis and J. Bodilis, in preparation).

Finally, in addition to changing or diversifying their pyoverdine and their associated FpvA receptor, acquisition of alternative receptors (without the PVD genes), like FpvB but also like the 35 other putative TonB-dependent receptors identified in the PAO1 genome (Cornelis *et al.*, 2007), can be considered as a cheap (and cheat) strategy to increase the fitness.

The MPCR described in this study allows a more rapid and accurate identification of the pyoverdine type compared with the IEF-based method for siderotyping (Meyer *et al.*, 1997) and should also be useful for the typing of pyoverdine-negative strains that are often isolated from Cystic Fibrosis (CF) lungs (De Vos *et al.*, 2001). Since nine patterns are possible (*fpvA*I, *fpvA*IIa, *fpvA*IIb, *fpvA*III, *fpvA*I-*fpvB*, *fpvA*IIa-*fpvB*, *fpvA*IIb-*fpvB*, *fpvA*III-*fpvB*, *fpvB*), this MPCR could be useful as a complementary technique for typing *P. aeruginosa* isolates. Since it appears that several typing methods, with different degrees of resolution, are necessary for the study of *P. aeruginosa*, similar MPCR assays could be designed by including other receptor genes, such as *fptA* for pyochelin (Ankenbauer and Quan, 1994) or *pfeA* and *pirA* for ferrienterobactin (Ghysels *et al.*, 2005).

Experimental procedures

Bacterial strains used in this study

The *P. aeruginosa* strains used for reference in this MPCR are PAO1, a type I pyoverdine producer (Stover *et al.*, 2000), 7NSK2 and ATCC27853, both type II pyoverdine producers (De Chial *et al.*, 2003), and 59.20 as an example of a type III pyoverdine producer (De Chial *et al.*, 2003). Some (75 strains) of the 345 strains used in this study are reported in Fig. 2. A list of all the strains used for this study as well as their origin is available in Table S1.

Primers and PCR conditions

The primers used for this MPCR are listed in Table 3. The PCR was performed using ™Ex-Taq polymerase (Takara), supplied with buffer and dNTPs, according to the following cycling parameters: 94°C (5 min) followed by 30 cycles [94°C (30 s)–52°C (30 s)–72°C (2 min)] and a final extension [72°C (10 min step)]. All the primers were manufactured by Eurogentec (Seraing, Belgium). The template for the PCR-mix was either a pipette tip of bacterial cells (without prior boiling), or 2 μl of a chromosomal DNA preparation. Double-stranded DNA sequencing of some *fpvA* and *fpvB* genes was carried out by the VIB sequence facility. The nucleotide sequences determined in this study have been deposited in the GenBank database.

Table 3. Primers used in this study.

Primer	Position	Expected size (bp)	Sequence
*fpvA*If	1833		5′-CGAACCCGACGAAGGCCAGA-3′
*fpvA*Ir	2157	324	5′-GTAGCTGGTGTAGAGGCTCAA-3′
*fpvA*IIaf	658		5′-TACCTCGACGGCCTGCACAT-3′
*fpvA*IIar	1566	908	5′-GAAGGTGAATGGCTTGCCGT-3′
*fpvA*IIbf	865		5′-GAACAGGGCACCTACCTGTA-3′
*fpvA*IIbr	1728	863	5′-GATGCCGTTGCTGAACTCGTA-3′
*fpvA*IIIf	1276		5′-ACTGGGACAAGATCCAAGAGA-3′
*fpvA*IIIr	1781	505	5′-CTGGTAGGACGAAATGCGA-3′
*fpvB*f	1561		5′-GCATGAAGCTCGACCAGGA-3′
*fpvB*r	2123	562	5′-TTGCCCTCGTTGGCCTTG-3′

Phylogenetic analyses

From 22 strains (including the seven strains for which the genomes were sequenced), nearly complete FpvA and/or FpvB sequences (41 sequences in total) were aligned using CLUSTALX version 1.81, with default parameters (Thompson *et al.*, 1997), and optimized visually. The nucleic acid alignment was deduced from the corrected protein alignment, leading to about 2300 aligned nucleotide positions.

A set of 34 ubiquitous ribosomal genes were retrieved from the seven (fully or partially) sequenced *Pseudomonas aeruginosa* genomes (PAO1, LES, 2192, PACS2, C3719, PA7 and PA14 strains). All the genes were aligned individually and concatenated, leading to 12 735 unambiguously aligned nucleotide positions.

From nucleic alignments, Bayesian analysis was performed using MrBayes 3.1 (Ronquist and Huelsenbeck, 2003). The Modeltest software (Posada and Crandall, 1998) was used to choose the evolutionary model. For both phylogenies (PVD receptor and ribosomal genes), the model used is the complex GTR with an among-site rate heterogeneity (GTR + γ). In addition, we also used a model that takes into account rate heterogeneity among positions in codon. Since the resulting topologies were identical for the two models, except for two weak-supported nodes in the *fpvB* cluster, only the phylogenetic analyses from the first model were presented in Fig. 5. All analyses were carried out with random starting trees. Four Metropolis coupled Markov chain Monte Carlo (MCMC) chains were run, stopping after 1 or 2 million generations (for ribosomal and PVD receptor genes respectively), when the standard deviation of split frequencies was less than 0.01. Trees were sampled every 100 generations and the first 25% burn-in cycles (i.e. 2500 or 5000 trees) were discarded prior to consensus trees construction. Analyses were repeated twice to ensure the correct topology. Consensus trees were visualized with TreeView 1.6.6 (Page, 1996) and posterior probabilities were employed to test the statistical support of clades. Additionally, a data set consisting of the AFLP pattern, *oprI*, *oprL* and *oprD* gene sequences, and serotype of 75 *P. aeruginosa* isolates was analysed using biological data analysis software. AFLP band patterns were imported into BioNumerics v5.2 software (Applied Maths, Belgium) for further normalization (background subtraction, filtering: arithmetic average, and band search: minimum profiling 0.5% relative to maximum value) and cluster analysis (similarity coefficient: Pearson correlation, dendrogram type: UPGMA, optimization: 0%, position tolerance: 1%, uncertain bands were ignored). Sequences were clustered (Pairwise alignment, open gap penalty: 100%, unit gap penalty 0%, minimum match sequence: 2, maximum number of gaps: 9, fast algorithm), aligned (multiple alignment, open gap penalty: 100%, unit gap penalty: 0%, minimum match sequence: 2, maximum number of gaps: 98) and clustered a second time (using the same parameters) using BioNumerics v5.2 software. The serotypes were compared using the Pearson correlation. These individual comparisons resulted in individual similarity matrices. These similarity matrices were averaged into the similarity matrix of the composite data set. No correction for internal weights was applied. A dendrogram (UPGMA, BioNumerics v5.2) based on the comparison of the composite data set was built.

Sequence analyses

The synonymous and non-synonymous rates were determined using the modified Nei-Gojobori method implemented in the MEGA v2.0 software (Kumar *et al.*, 2001). The transition to transversion ratio was fixed at 2 and the Jukes–Cantor correction was used to account for multiple substitutions at the same site. Codon adaptation index (CAI) was calculated with the new method implemented in DAMBE software which deals with several computational problems (Xia and Xie, 2001; Xia, 2007). All the measurements were also carried out with the classical method as implemented in EMBOSS.cai program (Rice *et al.*, 2000) and, although the values were always lower than the ones presented here, the trends were the same (data not shown). As CAI is a measure of the relative codon usage bias of a gene towards the average codon usage of an organism, a reference codon usage table of the given organism is required. Because only the reference codon usage table of the PAO1 strain is available in EMBOSS and DAMBE data (Epae), we wondered whether differences in codon usage between *P. aeruginosa* strains would prevent us using the same reference table for all *P. aeruginosa* strains. To deal with this problem, we estimated seven reference codon usage tables from concatenated ribosomal genes of the seven *P. aeruginosa* sequenced genomes, by using the *cusp* program of EMBOSS (Rice *et al.*, 2000). Next, we used these reference codon usage tables to calculate CAI (with classical and new methods) for several genes and found almost identical results, whatever the strains used to construct the reference codon usage tables (data not shown), highlighting almost identical optimal codon usage between the different *P. aeruginosa* strains tested. Therefore only the results obtained with the reference codon usage table of the PAO1 strain (Epae) are presented here.

Pyoverdine typing by IEF

For IEF typing, pyoverdines were partially purified by chromabond C18-affinity chromatography from 10 ml supernatant of cell culture in casamino acid medium (CAA). Pyoverdine was eluted from this matrix with a 1:1 water/methanol mixture. Pyoverdine-IEF was carried out on Ampholine PAG plates (pH 3.5–9.5; Pharmacia) as described previously (Meyer *et al.*, 1997). For growth stimulation assays, pyoverdines from the different reference strains (PAO1, 7NSK2 or 59.20) were semi-purified on a preparative scale on an XAD-4 amberlite column as described earlier (Budzikiewicz, 1993; Ghysels *et al.*, 2004).

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Strains used in this study, indicating their MPCR results, origin, year, and source (environmental strains in yellow).

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